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Full Length Research Paper

Comparison of Agar screen and duplex-PCR methods in determination of methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from nasal carriage

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Methicillin-resistant *Staphylococcus aureus* strains (MRSA) have become a serious health issue in engendering nosocomial infections. Due to the heterogeneity of this type of resistance, the conventional antibiotic susceptibility tests may fail to detect MRSA strains. The purpose of this research was to compare the phenotypic agar screen method with polymerase chain reaction (PCR) for detection of MRSA strains isolated from the nasal samples of hospital personnel. Totally, 52 coagulase positive *S. aureus* strains were isolated from nasal samples of 204 hospital personnel of Hajar Hospital affiliated to Shahrekord University of Medical Sciences. Susceptibility to oxacillin in the strains was evaluated by the phenotypic agar screen method. The presence of the methicillin resistance gene, *mecA*, was studied through duplex PCR method. The results of both methods were compared and the sensitivity and specificity of the methods were determined. Totally, 23 out of the 52 isolated *S. aureus* (44%) were phenotypically resistant to oxacillin, but 27 (52%) carried *mecA* gene. The sensitivity and specificity of the phenotypic agar screen method for determination of MRSA strains were found to be 81.5 and 96%, respectively. As compared to duplex PCR, oxacillin agar screen method is a simple, inexpensive, and practical phenotypic method with relatively low false positive results and thus may be suitable for verification of suspicious MRSA strains. However, for the relatively high false negative results, it may not be recommended for the primary screening of MRSA strains from the nasal samples of healthy carriers working at hospitals.

Key words: Nosocomial infections, methicillin resistance *Staphylococcus aureus* (MRSA), polymerase chain reaction (PCR).

INTRODUCTION

During the last 40 years, Methicillin-resistant *Staphylococcus aureus* strains (MRSA) infections have

become endemic in the hospitals of different regions of the world (Diekema et al., 2001). In the USA, from every two million nosocomial infections annually, 26000 cases are caused by *S. aureus* strains. There has been a pronounced increasing trend in the frequency of MRSA strains from 14.8% in 1987 to 39.7% in 1998 (Sakoulas et al., 2001). *S. aureus* is an important cause of serious

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infections of the skin and soft tissues and invasive infections that arise from hospital or society (NNIS, 2002).

Resistance of *S. aureus* strains to penicillin was first reported only a year after its discovery (Deresinski, 2005). The resistance was linked to beta lactamase enzyme, encoded by the plasmid. The spread of resistance was so quick that made the treatment of the resulting infections difficult. In 1960, the production of the semi-synthetic penicillinase-resistant penicillin (PRP), also named as methicillin, was acknowledged to be quite promising for a successful treatment of the related infections. However, after less than a year, MRSA strains were reported in the UK (Kim, et al., 2004). These strains were first detected in hospitals but soon were also reported from the society (Deresinski, 2005).

The resistance to penicillin in *S. aureus* is heterogeneous in nature in such a way that less than one in 100 cells express the resistance gene at a high level (Sakoulas et al., 2001). The mechanism of this heterogeneous resistance found in MRSA strains is not completely known. Although, we do know that the existence of chromosomal *mecA* gene, coding for PBP2a protein is necessary for developing the resistance (Kim et al., 2004; Sakoulas et al., 2001) and also that two groups of factors are involved in the regulation of its expression; the first group includes the genes such as *femA* that are involved in peptidoglycan synthesis; The second group of factors are environmental conditions such as osmolarity of culture medium (for example, NaCl density), temperature, and incubation time span (Berger-Bachi et al., 1989; Hiramatsu et al., 1990; Ryffel et al., 1992; Sakoulas et al., 2001). Despite NCCLS recommendations for determining susceptibility of MRSA stains, it is quite possible that among a population of phenotypically methicillin-susceptible bacteria, a low percentage, some times less than one highly resistant cell per 10^8 cells, carry *mecA* gene. (Sakoulas et al., 2001). Therefore, it is difficult or even impossible to determinate susceptibility for all MRSA strains through routine microbiological methods (Brown et al., 2005).

Evidence shows that *mecA*-positive MRSA strains having heterogeneous resistance phenotype during incubation with methicillin progress toward homogeneously resistant strains (Kim et al., 2004). Furthermore, researches have shown that if *mecA*-positive MRSA isolates susceptible to methicillin are exposed to beta lactam antibiotics, their oxacillin minimum inhibitory concentration (MIC) will increase. Therefore, prescription of beta lactam antibiotics can cause selective pressure for the appearance of highly resistant bacteria among a population of susceptible bacteria resulting in treatment failures. Another important issue is the appearance of altered PBP2a proteins with little tendency to attach to antibiotics which result in resistance to a broad spectrum of antibiotics including penicillin, cephalosporin, carbapenem and also to beta lactamase resistant

semi-synthetic antibiotics such as methicillin, oxacillin and nafcillin (Deresinski, 2005). Unfortunately, most of staphylococci with heterogeneous resistance are resistant to numerous antibacterial agents such as beta lactamase, aminoglycosides, macrolides, clindamycin, and tetracycline (Nation Nosocomial Infections Surveillance., 2002; Wallet et al., 1996).

Since *mecA* gene is not found in staphylococci susceptible to methicillin (Wallet et al., 1996), PCR and hybridization- based molecular methods which determine *mecA* gene are considered to be gold standard methods (Brown et al., 2005; Wallet et al., 1996).

The present investigation was launched to determine the prevalence of MRSA strains isolated from the nasal samples of healthy carriers working at hospitals, as they are one of the potential sources of nosocomial infections (Diekema et al., 2001). Furthermore, we aimed to evaluate and compare the efficiency of oxacillin agar screen method with that of duplex PCR, as gold standard method.

MATERIALS AND METHODS

Bacterial isolates and bacteriologic methods

In this experimental method, 204 samples were collected since December, 2009 till December, 2010 from anterior part of noses of volunteer personnel working in different wards of educational hospitals affiliated to Shahrekord University of Medical Sciences. *S. aureus* isolates were identified based on colonial morphology on the blood agar (Merck-German) plates, Gram stain characteristics, mannitol fermentation, catalase test, coagulase test, and DNase test agar (Gaillot et al., 2000).

Oxacillin Agar screen test

To determine susceptibility to methicillin, following NCCLS's recommendations, oxacillin was used, as it is more stable than methicillin in the lab conditions and is able to recognize cross-resistance. In addition, agar screen method was used which is preferable to the disk diffusion method (NCCL, 2000). Susceptibility determination was performed by inoculation of CFU of 10^4 bacteria on Muller-Hinton agar (Merck-German) including 4% sodium chloride and 6 µg/ml oxacillin (Merck, German). Bacterial growth was checked after 24 h incubation at 35°C (NCCL, 2000).

mecA duplex PCR

In order to detect the *mecA* gene, the methicillin resistance gene, two specific pairs of primers were used (Sina Gene Co, Iran) (Table 1). These primers amplify a 310 bp fragment of *mecA* gene and a 479 bp fragment of 16S rRNA gene. The latter amplicon served as an internal quality control of PCR reactions (Geha et al., 1994; Jonas et al., 2002).

To extract bacterial genomic DNA, Diatom DNA Prep kit (IzoGen, Russia) was used. Standard cycling conditions were followed using a thermocycler (ASTEC PC818-Japan). In a total reaction volume of 25 µl, 1 µl template DNA (about 30 ng), 1 µl of each primer (10 pmol/ µl), 0.5 µl dNTP mix (10 mM), 2.5 µl PCR buffer (1x), 2 µm MgCl₂ (50 mM) and 0.1 µl (5 U/ul) of Taq DNA polymerase were used. The DNA amplification consisted of an initial denaturation step (96°C, 4 min), followed by 30 cycles of denaturation (96°C,

40 s), annealing (59°C, 40 s) and extension (72°C, 1 min), and a single final extension of 5 min at 72°C. *S. aureus* (ATCC 29213) was used as the negative control for *mecA* gene and *S. aureus* (ATCC 33591) was used as the *mecA* positive control.

Statistical analysis

Results were analyzed using SPSS (version 13, SPSS, Chicago). Differences in susceptibility methods and the significance of the results were calculated by the Chi-square test or Fisher exact test. The *P* value of < 0.05 was considered to be statistically significant. Validity tests including susceptibility, specificity, positive predictive value, and negative predictive value were calculated. Susceptibility was defined as the percentage of *mecA*-positive isolates determined to be susceptible by phenotypic testing and specificity was defined as the percentage of *mecA*-negative isolates determined to be susceptible by phenotypic testing. The 2006 CLSI criteria were used to determine susceptibilities.

RESULTS

Out of 204 isolated staphylococci, 52 strains were coagulase positive (25.5%), among which 23 strains (44%) showed resistance to oxacillin using phenotypic agar screen method. Results of duplex PCR method showed 27 strains (52%) to be positive for *mecA* gene (Figure 1). Therefore, out of the 27 coagulase positive strains having *mecA* gene, only 22 (81.48%) were identified as being resistant to methicillin via the phenotypic agar screen method. Thus, 5 strains having *mecA* gene were not detected through phenotypic agar screen and one strain devoid of *mecA* gene was identified as resistant to oxacillin (Table 2).

The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency of agar screen test were 81.5, 96, 95.6, 82.7, and 88.5%, respectively.

DISCUSSION

Frequency of the *mecA* gene among *S. aureus* strains has been reported differently from diverse parts of the world (Diekema et al., 2001; Sakoulas et al., 2001). Differences in the distribution of the *mecA* gene in various regions as well as using different methods among studies could account for the observation. However, an important issue raised by most of these studies is the fact that the *mecA* gene is globally distributed suggesting a potential threat by bacterial strains of *S. aureus* resistant to methicillin as well as a wide spectrum of other antibiotics in the world. In this study, two methods for determination of MRSA strains including agar screen and duplex PCR were compared. Several studies have demonstrated that PCR for *mecA* detection is a highly sensitive, precise, and efficient method as compared to the usual phenotypic methods in MRSA recognition. PCR is as the gold standard since the presence of resistance gene itself is investigated through the methods (Dalla

Valle et al., 2009; Moussa and Shibl, 2009; Sakoulas et al., 2001).

Our study findings related to the existence of the gene in 52% of *S. aureus* strains isolated from the personnel working in Shahrekord hospitals, Southwest Iran, can be alarming. However, a study performed in Tabriz city, Northwest Iran, shows the prevalence of 21.33% (Hosain Zadegan and Menati, 2011). This suggests that inter regional differences exist in the country.

NCCLS evaluates *S. aureus* strains with MIC \leq 2 μ g/ml as susceptible and with MIC \leq 4 μ g/ml as resistant to methicillin (NIIS, 2002). In this research, results of phenotypic method showed 5 false susceptibility and one false resistance, comparable to the results obtained by other similar studies (Baddour et al., 2007; Resende and Figueiredo, 1997; Shariati et al., 2010; Swenson et al., 2001).

Heterogeneous resistance among MRSA strains may contributes to the failure of phenotypic methods to detect *mecA* positive strains which are mixed with the more frequent negative ones. In this regard, efficiency differences both among various methods and the same method under different circumstances may exist.

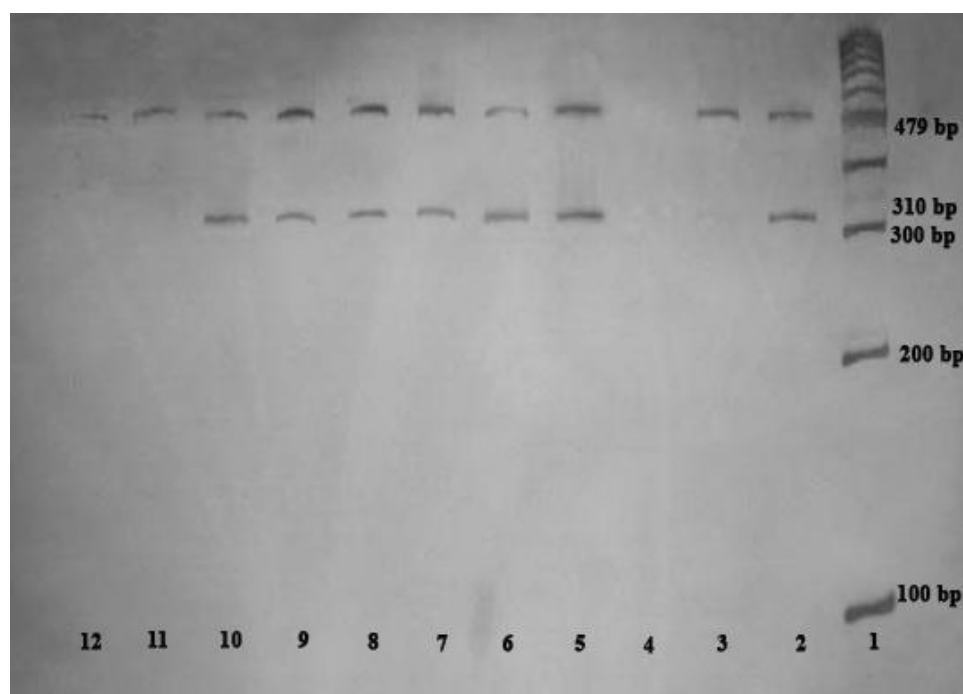
Although, oxacillin agar screen method, used in this research, was recommended by NCCLS, the sensitivity was as low as 81.5%, lower than the values reported for the technique (Al-Talib et al., 2010; Mimica et al., 2007; Shariati et al., 2010). This can be due to difference in strains or the nature of the samples. Sakoulas et al in their research concluded that heterogeneous resistance is different even in *S. aureus* isolates with various pathologic sources such as blood, mucus, wound and the eye (Cekovska et al., 2005; Sakoulas et al., 2001). Therefore, it might be concluded that agar screen has a low sensitivity for the primary screening of MRSA strains from the nasal samples of the healthy carriers. The relatively high false negative results can be due to its incapability to detect MIC \leq 4 μ g/ml strains which are evaluated as methicillin-susceptible.

On the other hand, in this research, one isolate devoid of *mecA* gene was observed to be phenotypically resistant to oxacillin. Other researchers have faced similar cases (Cekovska et al., 2005; Kolbert et al., 1995; Mulder, 1996; Shariati et al., 2010). Excessive production of beta lactamase or production of natural PBP proteins with altered ability for attachment and/or other unknown factors can cause low-level resistance to methicillin in *S. aureus* strains lacking *mecA* gene (Wallet et al., 1996).

In view of the false negative results obtained in this investigation, it seems that agar screen method has lower efficiency than MIC determining methods. Although molecular tests to detect *mecA* resistance gene are considered to be the gold standard, for several reasons including the requirement of special facilities, high cost, and skillful personnel, their exploitation may not be easy in any laboratory. Therefore, for routine tests, methods of MIC determination such as dilution serials, agar dilution

Table 1. Sequence of primers used in detection of *mecA* gene.

Situation	Sequences of 5' to 3'	Primer
318-342	GAT GAA ATG ACT GAA CGT CCG ATA A	<i>mecA</i> 1 (F)
603-627	CCA ATT CCA CAT TGT TTC GGT CTA A	<i>mecA</i> 2(R)
911-930	GGA ATT CAA ATG AAT TGA CGG GGG	16S rRNA (x)
1371-1399	CGG GAT CCC AGG CCC GGG ACC GTA TTC AC	16S rRNA (Y)

**Figure 1.** Polyacrylamide gel electrophoresis (PAGE) of the PCR products of *mecA* and 16S rRNA. Column 1: molecular weight marker (1 Kb ladder), column 2: positive control; column 3: negative control; column 4: blank; columns 5 to 10: *mecA* positive *S. aureus* strains; columns 11 to 12: *mecA* negative *S. aureus* strains.**Table 2.** Comparison of two phenotypic and genotypic methods in detection of oxacillin (methicillin)-resistant *Staphylococcus aureus*.

Duplex PCR Agar screen	<i>mecA</i> positive	<i>mecA</i> negative	Total
Resistant	22	1	23
Susceptible	5	24	29
Total	27	25	52

and E-test are appropriate alternatives for molecular methods and can be recommended.

The specificity of 96% gained for agar screen test in this study compared to PCR, and the simplicity and inexpensiveness of the test make it suitable for screening colonies that are isolated on daily basis and also for confirmation of suspicious resistant strains that are observed in disk diffusion tests.

Conclusion

Being a simple, inexpensive and practical phenotypic method that could be carried out in almost any lab and with relatively low false positive results, oxacillin agar screen (6 µg/L) method is appropriate for verification of suspicious methicillin-resistant strains. However, with its relatively high false negative results, it is not suitable for

primary screening of MRSA strains from nasal samples of healthy carriers working in hospitals and therefore, duplex PCR should be exploited. Based on our results, it seems that lower values of oxacillin than those recommended by NCCLS can lead to increased test sensitivity of the test.

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